

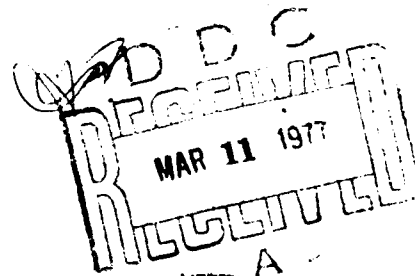
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The Exotoxin of P. aeruginosa: A Proenzyme Having an  
Unusual Mode of Activation

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Abstract. The exotoxin of Pseudomonas aeruginosa is a proenzyme possessing latent ADPR-transferase activity. Conversion to the active form can be effected by simultaneous treatment with a protein denaturant and a chemical able to split disulfide bonds. Activation results from a conformational change that exposes the previously buried active site. Proteolysis is not required.

The gram-negative bacterium Pseudomonas aeruginosa secretes a potent protein exotoxin which is lethal to a variety of experimental animals and cultured mammalian cells (1). Pseudomonas exotoxin (PE) may be an important virulence factor contributing to the high mortality of P. aeruginosa infections in patients having extensive burn wounds or agranulocytic malignancies (2). This toxin can be produced in quantities of a few hundred milligrams and purified to a state near homogeneity (3). PE joined the small group of protein toxins whose intracellular modes of action are known, when Iglewski and Kabat (4) showed that it has the same enzymatic activity as diphtheria toxin (DE). Both toxins catalyze transfer of the adenosine diphosphate ribose (ADPR) portion of nicotinamide adenine dinucleotide (NAD) to eukaryotic elongation factor 2 (EF-2). Modified EF-2 is unable to perform its normal translocase function in protein synthesis, so that toxin-exposed cells die.

Since PE has the same enzymatic activity as DE, we asked whether these two toxins have other similarities. One of the characteristic features of DE is its biosynthesis as an inactive proenzyme [reviewed by R. J. Collier (5)], which is rendered enzymatically active by cleavage into two large peptides, fragments A and B, through a process requiring peptide bond scission and reduction of a disulfide bridge. We show here that PE, like DE, is synthesized as a proenzyme, but that its activation can occur through a nonproteolytic process for which few precedents exist.

Except as noted below, our methods of preparing and analyzing PE samples were those previously described (3). The EF-2 substrate was a partially purified extract obtained from wheat germ by homogenization, precipitation with ammonium sulfate between 35 and 50 percent saturation, treatment with iodoacetamide, and batchwise chromatography on

diethylaminoethyl-cellulose. Rat liver EF-2, approximately 70 percent pure, was a gift of E. S. Maxwell, NIAMDD, Bethesda, Maryland.

Purified PE has a low level of ADPR-transferase activity which is not significantly increased by pretreatment with high concentrations of either dithiothreitol (DTT) or urea (Fig. 1). However, simultaneous exposure to DTT and urea causes a large increase in activity, approximately 100-fold in the sample tested. Several other preparations have shown 20- to 100-fold increases when "activated" in this manner. In experiments not detailed, we have shown that chemicals other than urea and DTT can also activate PE; increases in enzymatic activity equal to those seen in Fig. 1 are obtained when PE is treated simultaneously with any one of a number of protein denaturants [(urea, guanidine hydrochloride (GuHCl), sodium dodecyl sulfate (SDS)] and a reagent capable of breaking disulfide bonds (DTT, cysteine, 2-mercaptoethanol, sulfite). Activation can also be achieved by boiling the toxin in SDS and DTT, as might be expected from the ability of PE to retain enzymatic activity after apparently complete unfolding. These data demonstrate that PE is secreted in an enzymatically inactive, proenzyme form. Conversion to the active form appears to require simultaneous disruption of the secondary structure of the protein and cleavage of disulfide bonds.

The structural changes involved in activation of proenzymes fall into two classes, proteolytic and nonproteolytic. Nearly all the well characterized proenzymes, such as chymotrypsinogen and the blood clotting factors, are activated through proteolytic processes (6). DE is another example of a proteolytically activated proenzyme, one that is often overlooked in reviews of this subject (6). If proteolysis of PE were a prerequisite for activation it would seem to follow from Fig. 1 that

the purified PE had already experienced the necessary proteolysis. A precedent for such a situation is provided by DE, since a significant fraction of the molecules in most DE preparations possess cryptic proteolytic cleavages, or "nicks," between peptide fragments A and B. In these nicked molecules of DE a disulfide bridge holds the fragments together, thereby preventing expression of the latent ADPR-transferase activity of fragment A. The behavior of PE shown in Fig. 1 could be explained by a model similar to that of DE if it were proposed that the PE preparation is also highly nicked, but the disulfide bond linking the presumptive fragments lies buried in a hydrophobic environment inaccessible to reducing agents. Release of active fragment would thus require at least partial denaturation of the PE molecule for cleavage of the disulfide bond to occur. However, models involving nicking and production of large, enzymatically active fragments are inconsistent with previous analyses (3, 4) of PE by electrophoresis on polyacrylamide gels in SDS. Staining of gels for protein showed that PE behaves as a nearly homogeneous polypeptide of molecular weight 66,000. Prior treatment with reducing agent had no effect (4). Furthermore, at least 80 percent of the ADPR-transferase activity was associated with the 66,000 dalton species (3). In subsequent studies we have used tritium labeled albumin as an internal mobility marker in SDS gels to show that treatment with reducing agent does not cause a measurable change in the molecular weight of the enzymatically active species. It is estimated that a disulfide linked peptide fragment, if present, must have a molecular weight less than 2,000. These results make it unlikely that the activation phenomenon seen in Fig. 1 involves or requires proteolytic fragmentation of PE.

Though these results make proteolytic involvement improbable, they

do not rule out an alternative explanation for activation which also depends on proteolysis. This hypothesis suggests that reduced and denatured PE is not inherently active but becomes rapidly cleaved in the ADPR-transferase assay mixture to yield an active peptide fragment. Proteases able to cleave PE could be introduced with any of the ingredients of the reaction mixture, but the most likely source would be the relatively complex wheat germ extract added as a source of EF-2. The hypothesis that proteolysis occurring during the ADPR-transferase assay is necessary for activation predicts that the rate of reaction would increase as more active fragment is generated. However, when we measured the time course of the reaction at times (1 to 10 min) significantly below that routinely used (60 min), the rate was found to be constant and free of an initial lag, evidence that no processing essential to activation of PE occurs in the assay mixture. A more direct test became possible when we obtained a small sample of purified and presumably protease-free rat liver EF-2. Assays using this EF-2 gave results equivalent to those of Fig. 1, further indicating that proteolysis in the assay mixture is not required for activation.

Having effectively ruled out proteolytic explanations, it follows that activation must involve conformational rearrangement. PE is apparently synthesized by the bacterium in a conformational state in which all or part of the active site is sterically blocked. This conformation is maintained through a combination of hydrophobic forces and disulfide bonds. Activation, occurring when these stabilizing interactions are simultaneously disrupted, consists of an unfolding and subsequent conversion to a second relatively stable form in which the active site is available to the substrates NAD and EF-2.

To obtain direct evidence that activation correlates with unfolding of the protein and to determine whether scission of all four of the disulfide bonds of PE is required to expose the active site, we measured these processes as a function of denaturant concentration (Fig. 2). PE samples were incubated in 2 mM DTT and various concentrations of guanidine hydrochloride and then divided into two portions. One of these was reacted with [ $^{12}\text{C}$ ]iodoacetamide, diluted, and assayed for ADPR-transferase activity. (Studies to be reported elsewhere have shown that blocking all the half-cystines of PE with iodoacetamide does not decrease enzymatic activity.) The other portion was reacted with [ $^{14}\text{C}$ ]iodoacetamide to quantitate the newly formed sulfhydryls. The data fail to demonstrate that a subset of particularly labile disulfide bonds exists. Instead it appears that unfolding is a concerted event, that in the presence of a low concentration of DTT the PE molecule is rather easily denatured (ribonuclease is typical of most proteins in its resistance to guanidine denaturation), and that unfolding of the protein correlates with activation. The valley in ADPR-transferase activity at 1.0 M GuHCl has been seen in three experiments, suggesting that intermediate concentrations of denaturant may allow disulfide bond interchange or trapping of partially denatured forms. The results of this experiment support the view that it is the unfolding of PE which causes activation. The apparent ease with which PE can be unfolded suggests that this process might occur under physiological conditions, perhaps with a hydrophobic region of the cell membrane acting as denaturant and the glutathione present in the cytoplasm serving to break the disulfide bonds.

While activation by the unfolding mechanism described here is sufficient to explain the toxicity of PE, there is also evidence that an



enzymatically active peptide fragment can be formed. In work to be described elsewhere, we, as well as others (7), have found that activated PE can be fragmented to yield an enzymatically active species approximately the size of fragment A of DE. Conditions for in vitro fragmentation of PE have not yet been found which achieve the high specificity seen in the nicking of DE; a variety of peptide fragments are formed and some of the enzymatic activity of the sample is destroyed. Current evidence therefore suggests that PE can be activated in vitro by two procedures: unfolding induced by reduction and denaturation as described here, or proteolytic fragmentation like that involved in activation of DE. A precedent for this situation is provided by Hageman factor (Factor XII), a protease involved in initiating cascades leading to production of fibrin, kinin, and plasmin (8). Hageman factor, synthesized as an intact polypeptide of molecular weight 80,000, can be activated through two distinct mechanisms: (1) nonproteolytic activation due to a conformational change induced by interaction with substances, such as kaolin or glass, that have a high density of negative charges, and (2) proteolytic activation by trypsin, plasmin, or kallikrein causing production of an enzymatically active fragment of molecular weight 28,000. Both routes produce an active protease. It is not known whether one or both of these mechanisms occur under physiological conditions.

Models for in vivo activation of DE have been based in part on the requirement for separation of fragment A from fragment B (9). It is suggested that the hydrophobic B region interacts with the membrane in such a way that the arginine-rich sequence joining A to B becomes exposed on the inner surface of the membrane. Cytoplasmic proteases then nick the DE and the disulfide bond is broken by reducing agents, releasing

fragment A into the cytoplasm. Since PE can apparently be activated in vitro through either proteolytic or nonproteolytic routes, no analogous argument can be made regarding its in vivo mechanism of activation. Careful analysis of the cytoplasm of toxin-treated cells will be necessary to determine whether the unfolded protein or the proteolytic fragment is the active intracellular agent.

The structure of PE described here contributes another example supporting the emerging view that toxic proteins consist of two types of subunits or peptide regions, one ("A") which acts as the effector moiety and another ("B") which plays a role in binding of toxin to sensitive cells. Examples which seem to fit this pattern now include PE, DE, cholera, tetanus, and botulinum toxins, and the lectins, abrin and ricin (10). In those cases where the effector moiety has been found to act enzymatically (PE, DE, and cholera toxin), that enzymatic activity is not expressed in the native toxin, apparently because the B portion blocks the active site. In the continuing attempts to identify enzymatic activities in the less well characterized toxic proteins, it should be recognized that these presumptive proenzymes may require unusual treatments to achieve activation, as has been illustrated here by the example of PE.

## Figure Legends

Fig. 1. Effect of prior reduction and denaturation on ADPR-transferase activity of Pseudomonas exotoxin. Aliquots of 5  $\mu$ l of a 7 mg/ml solution of Pseudomonas exotoxin (PE) were transferred to four conical plastic vials which were either empty (NONE), or contained 5 mg urea (UREA), 2  $\mu$ l 50 mM dithiothreitol (DTT), or both urea and dithiothreitol (UREA + DTT). The vials were agitated gently for 10 minutes at 23°C to dissolve the urea. To each vial was then added 0.35 ml 50 mM tris, 5 mM EDTA, pH 3.1. Aliquots were diluted serially 10-fold in the same buffer and duplicate ADPR-transferase assays were performed on 15- and 50- $\mu$ l portions.

Fig. 2. Activation of ADPR-transferase and reduction of disulfide bonds in P. aeruginosa exotoxin (PE) as a function of guanidine hydrochloride (GuHCl) concentration. Samples of PE and ribonuclease (RNase) at 200  $\mu$ g/ml were incubated for 90 minutes in 50 mM tris, 5 mM EDTA, 2 mM dithiothreitol (DTT), pH 8.5, containing the concentrations of GuHCl shown on the abscissa. All incubations were performed at 23°C.

A. For assay of ADPR-transferase activity, 20- $\mu$ l aliquots of PE solutions were mixed with 2  $\mu$ l of 50 mM iodoacetamide, incubated 15 minutes in the dark, diluted 1000-fold in 50mM tris, 5 mM EDTA, 0.01 percent bovine albumin, pH 8.5, and assayed in duplicate on 10- $\mu$ l portions.

B. To measure newly formed sulphydryl groups, duplicate 20- $\mu$ l aliquots of the PE and RNase solutions were mixed with 2  $\mu$ l of 50 mM [1-<sup>14</sup>C]iodoacetamide (approximately 1.8 mCi/mmol), incubated 15 minutes in the dark and transferred to paper discs which were processed to determine trichloroacetic acid precipitable radioactivity as previously described (3).

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Fig. 8  
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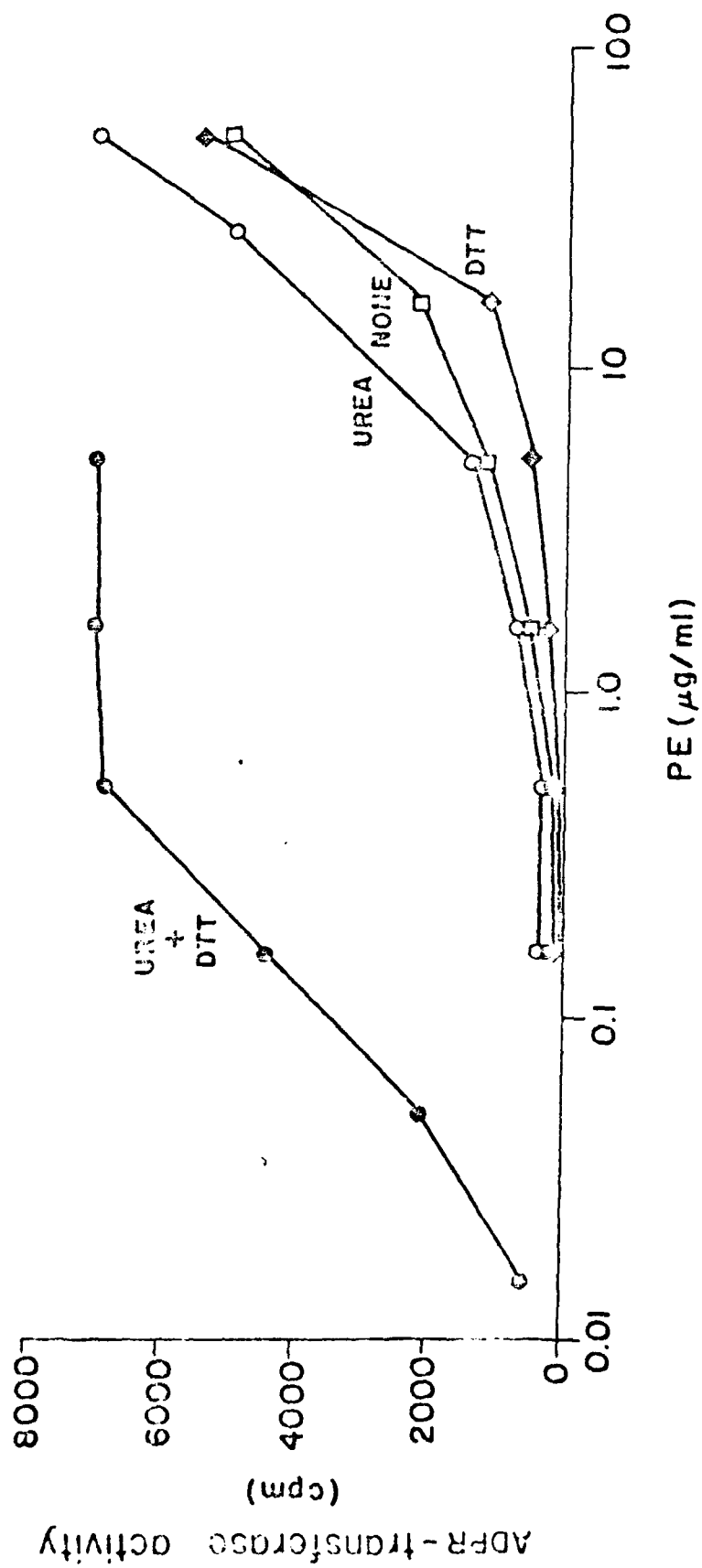


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